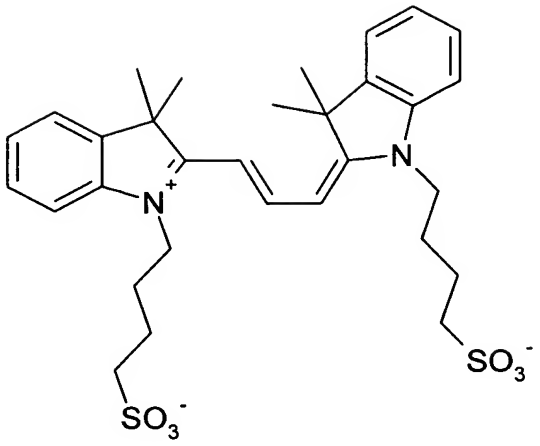

The Specification

The new matter issue raised regarding the specification is moot. The above structure clearly demonstrates that Y being $=C(CH_3)_2$ is not new matter.

Claim Rejections Under 35 U.S.C. § 103

The Office Action assumes common ownership at the time the invention was made for purposes of section 103. Applicants confirm this assumption.

The claims are rejected as allegedly obvious over Neri (Nature Biotechnology), in view of Viti, applicants' alleged admission in the specification, and Licha.

The Office Action alleges that Neri et al. show that the cyanine dye CY-3 does not affect the binding affinity of scFv while pointing to page 1273, column 1, third and fourth full paragraphs and figure 3. However, this is irrelevant as to how the dye of the present claims' antibody-dye conjugate would behave, e.g., affect the binding affinity of the antibody. At most the alleged disclosure by Neri is relevant to the specific combination tested by Neri. One of ordinary skill in the art would not extrapolate said specific teachings to all possible dyes, including the dyes subject of the present claims.

The arguments from the previous Reply are incorporated herein.

Reconsideration is respectfully requested.

Various claims are rejected as allegedly obvious over Neri (WO 99/58570), in view of additional references in two separate rejections.

Applicants attach a verified translation of the priority document and apologize for the filing error with the last reply where the verified translation of the priority document was omitted.

The claims are rejected as allegedly unpatentable over the same references as in the first rejection as above and further in view of Licha '570.

The arguments from above are incorporated herein. Applicants submit that '570 does not cure the deficiencies of the references already cited in the rejection above.

Licha teaches short-chain peptide and dye conjugates and not antibody-dye conjugates as claimed. Nothing in Licha teaches or motivates one of ordinary skill in the art to formulate an antibody-dye conjugate according to the claims. Instead, Licha teaches that the compounds (conjugates) are receptor-specific and allow for the diagnosis of tumor cells and tissues which express somatostatin receptors, etc. See first full paragraph on column 2. Nothing in Licha teaches or suggests the use of the compounds taught therein, or separate components thereof, e.g., the dye in the different combination of this invention for the visualization of an edge area of a disease. As such, this reference also does not provide adequate motivation to one of ordinary skill in the art, alone or in combination with the other prior art references, to render the presently claimed invention obvious.

Double Patenting Rejections

The Office Action alleges that the reference's conjugate containing "a protein, peptide, nucleic acid, etc., linked to the cyanine dye" renders the current claims obvious under obviousness-type double patenting. While the terms "protein, peptide, nucleic acid, etc." are generic and may contain antibodies, such is not adequate to render the present claims unpatentable for double patenting. There has to be obviousness. Since there is not even an allegation of obviousness, this rejection should be withdrawn.

The Commissioner is hereby authorized to charge any fees associated with this response or credit any overpayment to Deposit Account No. 13-3402.

Respectfully submitted,

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Design and Use of a Phage Display Library

HUMAN ANTIBODIES WITH SUBNANOMOLAR AFFINITY AGAINST A MARKER OF ANGIOGENESIS ELUTED FROM A TWO-DIMENSIONAL GEL*

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We report the construction and the use of a phage display human antibody library (>3 × 10^8 clones) based on principles of protein design. A large repertoire of functional antibodies with similar properties was produced by appending short variable complementarity-determining region 3 (CDR3) onto the two antibody germ line segments most frequently found in human antibodies. With this strategy we concentrated sequence diversity in regions of the antibody structure that are centrally located in the antigen binding site, while leaving residues in more peripheral positions available for further mutagenesis aimed at improving the affinity of the selected antibodies.

In addition, the library was tested by selecting antibodies against six biologically relevant antigens. Using only 0.3 μg of antigen eluted from a two-dimensional gel spot, we isolated binders specific for the ED-B domain of fibronectin, a marker of angiogenesis. These antibodies recognize the native antigen with affinities in the 10^7–10^8 M^{-1} range, and perform well in immunosorbent assays, in two-dimensional Western blotting and in immunohistochemistry. The affinity of one anti-ED-B antibody was improved by 27-fold by combinatorially mutating six strategically selected residues in the heavy chain variable domain. A further 28-fold affinity improvement could be achieved by mutating residues 32 and 50 of the light chain. The resulting antibody, L19, bound to the ED-B domain of fibronectin with very high affinity (K_d = 54 pM), as determined by real-time interaction analysis with surface plasmon resonance detection, band shift analysis, and by competition experiments with electrochemiluminescent detection.

As the era of intensive gene discovery approaches its conclusion, major efforts are under way to develop systematic means for the discovery of gene function and regulation. Advanced technologies in this field will be particularly useful if they operate at the level of the genes' functional products: the proteins. The efficient description of the protein phenotype in complex biological samples may allow unraveling of the protein composition of cells and tissues and analysis of diseases (as well as the effects of drugs and other xenobiotics) in terms of changes in protein composition (1).

Two-dimensional gel polyacrylamide electrophoresis (two-dimensional PAGE)[1] is arguably one of the most powerful methodologies for the description of protein phenotypes (2, 3), because it can separate and quantitate thousands of individual proteins from complex biological samples. The potential of two-dimensional PAGE would be extended if good quality affinity reagents (*e.g.* antibodies) could be produced against individual spots from gels. Such reagents could provide the structural information that is typically lost during treatment of biological samples needed for electrophoresis (total protein preparations are often obtained by lysing and homogenizing cells or tissues). The production of antibodies against gel spots would for example allow the microscopic analysis of the proteins in structurally intact samples (*e.g.* by immunofluorescence or immunohistochemistry) and the development of immunometric assays for the rapid quantitation of proteins in different specimens. The large number of potentially useful markers identified by two-dimensional PAGE analysis, however, greatly outstrips the capacity of conventional methods for monoclonal antibody production.

Phage antibody technology (4, 5) could in principle offer a solution, as the methodology is simple, inexpensive, and lends itself to simultaneous processing of several antigens. Relatively large amounts of pure native proteins have so far been used for the isolation of antibodies from naive phage libraries (5–10), although a few direct phage antibody selections on cells have been described (11–14). Recent advances in sample application (15) have allowed the application on two-dimensional gels of up to 1-ml samples, containing up to 10 mg of protein, without significant loss of resolution. As a consequence, microgram quantities of individual antigens are available in practice for antibody production. This poses stringent requirements on the quality of antibody phage display library and on the selection strategy.

We have therefore aimed at producing a large functional antibody library, that could reliably yield good quality antibodies against relevant biological markers. Because for several biological applications high-affinity binders are needed, the phage library should be constructed in a way that allows the facile affinity maturation of antibodies of interest. We have also

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§ These two authors contributed equally to the work.

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[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; CDR3, complementarity-determining regions 3; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonium]-1-propanesulfonic acid.

aimed at developing a selection methodology that could work with minute amounts of antigen, such as proteins eluted from two-dimensional gel spots.

We describe the production of a robust antibody phage library (>300 million clones), constructed using principles of protein design. We successfully selected antibodies against six biologically relevant proteins, including the ED-B domain of fibronectin, a marker of angiogenesis (16–18), eluted from a two-dimensional gel spot. These antibodies performed well in biochemical applications such as immunosorbent assays, two-dimensional Western blotting, and immunohistochemistry. Thanks to the modular design of the antibody library, one anti-ED-B antibody could easily be affinity-matured, yielding a binder with dissociation constant in the picomolar range (K_d = 54 pM).

EXPERIMENTAL PROCEDURES

Library Construction and Cloning—The antibody library was cloned using single VH (DP47; Ref. 19) and Vk (DPK22; Ref. 20) germ line genes (see Fig. 1 for the cloning and amplification strategy). Antibody residues are numbered according to Ref. 34. The VH component of the library was created using partially degenerate primers (Fig. 1 and Table I) in a PCR-based method to introduce random mutations at positions 95–98 in CDR3. The VL component of the library was generated in the same manner, by the introduction of random mutations at positions 91, 93, 94, and 96 of CDR3. PCR reactions were performed as described (6).

VH-VL scFv fragments were constructed by PCR assembly (Fig. 1; Ref. 21) from gel-purified VH and VL segments, double-digested with *NcoI/NotI*, and ligated into 15 μg of *NotI/NcoI*-digested pDN332 phagemid vector.

pDN332 is a derivative of phagemid pHEN1 (7), in which the sequence between the *NotI* site and the amber codon preceding the gene III has been replaced by the following sequence, coding for the D_3SD_2-FLAG-His_6 tag (22).

*Not*1	D	D	D	S	D	D	D	Y	K	D	D	D	D	K	H	H	H	H	H	amber
5′-GCG GCC GCA	GAT	GAC	GAT	TCC	GAC	GAT	GAC	TAC	AAG	GAC	GAC	GAC	GAC	AAG	CAC	CAT	CAC	CAT	CAC	CAT	TAG-3′

SEQUENCE 1

Transformations into TG1 *Escherichia coli* strain were performed according to Marks *et al.* (6), and phages were prepared according to standard protocols (9). Five clones were selected at random and sequenced to check for the absence of pervasive contamination. The percentage of clones that express folded antibodies was determined by immunoblot and dot-blot analysis using anti-FLAG M2 antibody (Eastman Kodak Co.) and anti-mouse horseradish peroxidase immunoglobulins (A2554; Sigma, Buchs, Switzerland) as detecting reagents or protein A-horseradish peroxidase as described (9). Protein A binds strongly to folded VH domains derived from the DP47 segment (23).

Library Selection—Recombinant fibronectin fragments ED-B and 7B89, containing one and four type III homology repeats, respectively, were expressed from pQE12-based expression vectors (Qiagen, Chatsworth, CA) as described (16).

Selections against recombinant ED-B domain of fibronectin (16, 17) were performed at 10 nM concentration using the antigen biotinylated with biotin disulfide *N*-hydroxysuccinimide ester (reagent B-4531; Sigma; Ref. 10) and eluted from a two-dimensional gel and captured using streptavidin-coated Dynabeads (10, 24).

10^{13} phages were used for each round of panning, in 1-ml reaction. Phage were incubated with antigen in 2% milk/PBS (MPBS) for 10 min. To this solution, 100 μl of Dynabeads (10 mg/ml; Dynal, Oslo, Norway), preblocked in MPBS, were added. After 5-min mixing, beads were magnetically separated from solution and washed seven times with PBS-0.1% Tween 20 (PBST) and three times with PBS. Elution was carried out by incubation for 2 min with 500 μl of 50 mM dithiothreitol, to reduce the disulfide bridge between antigen and biotin. Beads were captured again, and the resulting solution was used to infect exponentially growing TG1 *E. coli* cells.

After three rounds of panning, the eluted phage was used to infect exponentially growing HB2151. Soluble ELISA of bacterial supernatants obtained from single colonies was performed as described (9), using an anti-FLAG M2 monoclonal antibody (Kodak) as detecting reagent.

32% of screened clones were positive in this assay, and the three of them that gave the strongest ELISA signal (E1, A2, and G4) were sequenced and further characterized.

Selections against human tenascin-C (25), human chorionic somatotropin (Sclavo Diagnostics, Siena, Italy), tetanus toxoid (kind gift of Prof. Dr. Cesare Montecucco, University of Padova, Italy), Factor VIII (Sclavo Diagnostics), and interferon γ (kind gift of Dr. Gero Waschütza, Fraunhofer-IGB, Hannover, Germany) were performed in immunotubes (Maxisorp; Nunc, Roskilde, Denmark) coated with 25 μg/ml antigen as described (12).

Two-dimensional PAGE and Western Blotting—Two-dimensional electrophoresis was performed using a nonlinear immobilized pH gradient (range 3.5–10) in the first dimension with Immobiline strips (Pharmacia, Uppsala, Sweden). Approximately 45 mg of COLO-38 (26) and 1 μg of recombinant ED-B containing 7B89 (16) were loaded onto the gel. COLO-38 cells were pelleted by low speed centrifugation and resuspended in 8 M urea, 4% (w/v) CHAPS, 40 mM Tris base, 65 mM dithioerythritol. Recombinant 7B89 was used instead of ED-B because it is easier to blot (16) by virtue of its larger size.

A SDS-PAGE 9–16.5% (w/v) acrylamide gradient was used in second dimension (27), followed by protein transfer onto nitrocellulose membrane and immunodetection. The membrane was blocked with 2% MPBS for 2 h at room temperature. E1 anti-ED-B culture supernatant (see "Preparation of scFv Fragments") diluted 4:1 in 10% MPBS was then added (1.5-h incubation at room temperature). The membrane was washed twice for 5 min with 2% MPBS PBST and PBS only, then incubated with anti-FLAG monoclonal antibody (M2; Kodak) in 2% MPBS (0.5 μg/ml) for 1 h at room temperature, followed by horseradish peroxidase-conjugated anti-mouse IgGs (Jackon ImmunoResearch, West Grove, PA) in 2% MPBS (1:20,000 dilution). After washing as above, detection was obtained by ECL enhanced chemiluminescence (Amersham, Amersham, United Kingdom).

Biotinylated ED-B was run in two-dimensional PAGE, omitting dithioerythritol from the loading buffer, and eluted by fragmenting the gel piece and soaking in PBS overnight. Eluted protein was separated from the gel by centrifugation with a Millipore Ultrafree 0.45-μm microcentrifuge fil-tration device (Millipore, Yonezawa, Japan). The resulting solution was analyzed by gel electrophoresis to confirm protein purity after recovery and to quantitate ED-B concentration against standards at known concentration. Protein recovery using this methodology was approximately 50%.

Antibody Affinity Maturation—The gene of scFv(E1) was PCR-amplified with primers LMB1bis and DP47CDR1for (see Table I for primer sequences) to introduce random mutations at positions 31–33 in the CDR1 of the VH (for numbering, see Refs. 28 and 34) and with primers DP47CDR1back and DP47CDR2for to randomly mutate positions 50, 52, and 54 in CDR2 of the VH. The remaining fragment of the scFv gene, covering the 3′-portion of the VH gene, the peptide linker, and the VL gene, was amplified with primers DP47CDR2back and JforNot (94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min).

The three resulting PCR products were gel-purified and assembled by PCR (21) with primers LMB1bis and JforNot (94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min).

The resulting single PCR product was purified from the PCR mix, double-digested with *NotI/NcoI*, and ligated into *NotI/NcoI*-digested pDN332 vector. Approximately 9 μg of vector and 3 μg of insert were used in the ligation mix, which was purified by phenol extraction and ethanol precipitation, resuspended in 50 μl of sterile water, and electroporated in electrocompetent TG1 *E. coli* cells. The resulting affinity maturation library contained 4×10^8 clones.

Antibody-phage particles, produced as described (9), were used for a first round of selection on a B89-coated immunotube (16). The selected phages were used for a second round of panning performed with biotinylated ED-B, followed by capture with streptavidin-coated magnetic beads (Dynal, Oslo, Norway; see previous paragraph). After selection, approximately 25% of the clones were positive in soluble ELISA. From the candidates positive in ELISA, we further identified the one (H10;

Tables II and III) with lowest k_{off} by BIAcore analysis (29).

The gene of scFv(H10) was PCR-amplified with primers LMB1bis and DPKCDR1for to introduce a random mutation at position 32 in CDR1 of the VL (for numbering, see Ref. 28) and with primers DPKCDR1back and DPKCDR2for to introduce a random mutation at position 50 in CDR2 of the VL. The remaining portion of the scFv gene was amplified with oligonucleotides DPKCDR2back and JforNot (94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min).

The three resulting products were assembled, digested, and cloned into pDN332 as described above for the mutagenesis of the heavy chain. The resulting library was incubated with biotinylated ED-B in 3% bovine serum albumin for 30 min, followed by capture on a streptavidin-coated microtiter plate (Boehringer Mannheim GmbH, Mannheim, Germany) for 10 min. The phages were eluted with a 20 mM DTT solution (1,4-dithio-DL-threitol, Fluka) and used to infect exponentially growing TG1 cells.

Analysis of ED-B binding of supernatants from 96 colonies by ELISA and by BIAcore allowed the identification of clone L19.

Affinity Measurements—Affinity measurements by BIAcore were performed with purified antibodies as described (30). Band shift analysis was performed as described (22), using recombinant ED-B fluorescently labeled at the N-terminal extremity (16, 30) with the infrared fluorophore Cy5 (Amersham).

BIAcore analysis does not always allow the accurate determination of kinetic parameters for slow dissociation reactions because of possible rebinding effects, base line instability, and long measurement times needed to ascertain that the dissociation phase follows a single exponential profile. We therefore performed measurements of the kinetic dissociation constant k_{off} by competition experiments (31). In brief, anti-ED-B antibodies (30 nM) were incubated with biotinylated ED-B (10 nM) for 10 min, in the presence of M2 anti-FLAG antibody (0.5 μg/ml) and polyclonal anti-mouse IgG (Sigma), which had previously been labeled with a ruthenium complex as described (32). To this solution, in parallel reactions, unbiotinylated ED-B (1 μM) was added at different times. Streptavidin-coated Dynabeads, diluted in Origen assay buffer (32), were then added (20 μl, 1 mg/ml) and the resulting mixtures analyzed with a Origen analyzer (IGEN Inc., Gaithersburg, MD). This instrument detects an electrochemiluminescent signal (ECL) that correlates with the amount of scFv fragment still bound to the biotinylated ED-B at the end of the competition reaction. Plot of the ECL signal *versus* competition time yields a profile that can be fitted with a single exponential with characteristic constant k_{off}.

Preparation of scFv Fragments—Anti-ED-B antibody fragments were produced by inoculating a single fresh colony in 1 liter of 2 × TY medium as described previously in Pini *et al.* (33) and affinity-purified onto a CNBr-activated Sepharose column (Pharmacia), which had been coupled with 10 mg of ED-B containing 7B89 recombinant protein (16). After loading, the column was washed with 50 ml of equilibration buffer (PBS, 1 mM EDTA, 0.5 M NaCl). Antibody fragments were then eluted with triethylamine 100 mM, immediately neutralized with 1 M Hepes, pH 7, and dialyzed against PBS.

Immunohistochemistry—Immunostaining of sections of glioblastoma multiform samples frozen in liquid nitrogen immediately after removal by surgical procedures was performed as described (16, 18). In short immunostaining was performed using M2-anti-FLAG antibody (Kodak), biotinylated anti-mouse polyclonal antibodies (Sigma), a streptavidin-biotin alkaline phosphatase complex staining kit (BioSpa, Milan, Italy), and naphthol-AS-MX-phosphate and fast red TR (Sigma). Gill's hematoxylin was used as a counterstain, followed by mounting in glycergel (Dako, Carpenteria, CA) as reported previously (18).

RESULTS

Design, Construction, and Use of the Synthetic Antibody Phage Display Library—Human antibodies are assembled from 51 different VH germ line genes and 70 different functional VL segments (40 Vk and 30 Vλ; Refs. 34–36). However, one VH (DP47) and one Vk (DPK22) dominate the functional repertoire (37) and are well represented in binders isolated from synthetic phage libraries (10). Because our purpose was to generate a highly diverse library of functional antibodies with similar expression and performance, we opted to utilize only these two germ line gene segments, whereas randomizing four amino acid residues in the VL CDR3 (positions 91, 93, 94, and 96) and four residues in the VH CDR3 (positions 95–98; Fig. 1), in accordance with their role as common antigen contacts (38).

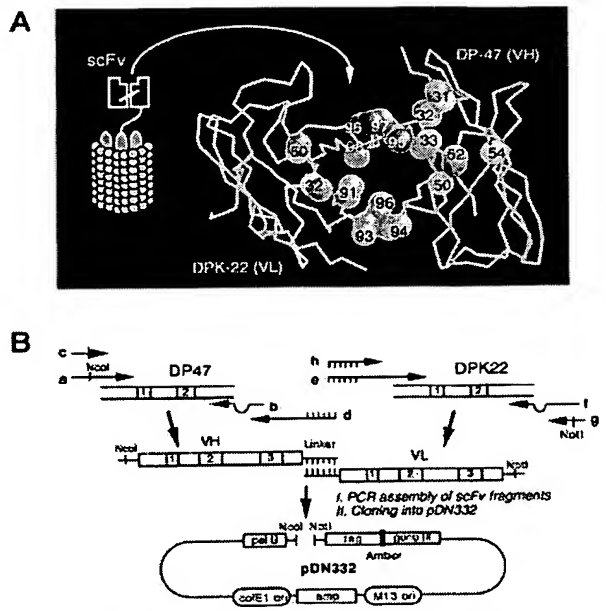

FIG. 1. **Designed antibody phage library.** *A*, antibody fragments are displayed on phage as pIII fusion proteins, as schematically depicted. In the antibody binding site (antigen's eye view), the Vk CDRs backbone is in *yellow* and the VH CDR backbone is in *blue*. Residues subject to random mutation are Vk CDR3 positions 91, 93, 94, and 96 (*yellow*) and VH CDR3 positions 95, 96, 97, and 98 (*blue*). The Cβ atoms of these side chains are shown in darker colors. Also shown (in *gray*) are the residues of CDR1 and CDR2, which can be mutated to improve antibody affinity. Using the program RasMol (http://www.chemistry.ucsc.edu/wipke/teaching/rasmol.html), the structure of the scFv was modeled from protein data base file 1igm (Brookhaven Protein Data Bank; http://www2.ebi.ac.uk/pcserv/pdbdb.htm). *B*, PCR amplification and library cloning strategy. The DP47 and DPK22 germ line templates were modified (see "Experimental Procedures") to generate mutations in the CDR3 regions. Genes are indicated as *rectangles* and CDRs as *numbered boxes* within the *rectangle*. The VH and the VL segments were then assembled and cloned in pDN332 phagemid vector. Primers used in the amplification and assembly are listed in Table I.

Like Hoogenboom and Winter (23) we did not randomize the structurally relevant and biologically conserved F-D-Y sequence at the end of the CDR3 VH (Fig. 1; Table I). Residues 92 and 95 of the VL CDR3 were kept constant (G and P, respectively), to allow for tight turn formation.

The antibodies were cloned in scFv configuration (39, 40) in a novel phagemid vector, pDN332, which appends at the C-terminal extremity of the recombinant antibody a described previously D_3SD_3-FLAG-His_6 versatile tag (22). Considering that eight amino acid positions were randomized, the potential diversity of the library is $20^8 = 2.56 \times 10^{10}$. In practice, because of limits in electroporation efficiency, our library consisted of $> 3 \times 10^8$ individual clones, tapping only a small fraction of the potential diversity. 88% of the clones expressed functional antibodies, as determined by Western blot and dot-blot analysis using anti-FLAG and protein A as detecting agents (see "Experimental Procedures").

To test the functionality of the library, we panned it against a number of antigens. The sequences of selected antibody clones isolated from the library are reported in Table II. Expression of antibody clones in shaker flasks typically ranged between 5 and 50 mg/liter of bacterial culture.

Antibody Selection from a Two-dimensional PAGE Spot—One of the challenges of panning phage libraries using proteins from two-dimensional PAGE spots is that selections and screenings have to be performed with minute amounts of antigen (typically

TABLE I

Oligonucleotide primers used for library construction and for antibody affinity maturation

The abbreviations a–h of oligonucleotides used for library construction are defined in the legend to Fig. 1.

		Synthetic antibody library primers:
VH primers		
a	DP47baNco	5′-GCG GCC CAG CAT GCC ATG GCC GAG GTG CAG CTG TTG GAG TCT GGG-3′
b	CDR3for	5′-GGT TCC CTG GCC CCA GTA GTC AAA MNN MNN MNN MNN TTT CGC ACA GTA ATA TAC G-3′
c	Vhpullth	5′-GCG GCC CAG CAT GCC ATG GCC GAG-3′
d	Jassm	5′-CCC GCT ACC GCC ACT GGA CCC ATC GCC ACT CGA GAC GGT GAC CAG GGT TCC CTG GCC CCA GTA GTC-3′
VL primers		
e	DPK22assm	5′-GAT GGG TCC AGT GGC GGT AGC GGG GGC GCG TCG ACT GGC GAA ATT GTG TTG ACG CAG TCT CC-3′
f	DPK3for	5′-CAC CTT GGT CCC TTG GCC GAA CGT MNN CGG MNN MNN ACC MNN CTG CTG ACA GTA ATA CAC TGC-3′
g	JforNot	5′-GAG TCA TTC TCG ACT TGC GGC CGC TTT GAT TTC CAC CTT GGT CCC TTG GCC GAA CG-3′
h	pullth	5′-GAT GGG TCC AGT GGC GGT AGC GGG-3′
		Affinity maturation primers:
LMB1bis		5′-GCG GCC CAG CCG GCC ATG GCC GAG-3′
JforNot		5′-TCA TTC TCG ACT TGC GGC CGC TTT GAT TTC CAC CTT GGT CCC TTG GCC GAA CG-3′
DP47CDR1for		5′-GAG CCT GGC GGA CCC AGC TCA TMN NMN NMN NGC TAA AGG TGA ATC CAG AGG CTG-3′
DP47CDR1back		5′-ATG AGC TGG GTC CGC CAG GCT CC-3′
DP47CDR2for		5′-GTC TGC GTA GTA TGT GGT ACC MNN ACT ACC MNN AAT MNN TGA GAC CCA CTC CAG CCC CTT C-3′
DP47CDR2back		5′-ACA TAC TAC GCA GAC TCC GTG AAG-3′
DPKCDR1for		5′-GTT TCT GCT GGT ACC AGG CTA AMN NGC TGC TGC TAA CAC TCT GAC TG-3′
DPKCDR1back		5′-TTA GCC TGG TAC CAG CAG AAA CC-3′
DPKCDR2for		5′-GCC AGT GGC CCT GCT GGA TGC MNN ATA GAT GAG GAG CCT GGG AGC C-3′
DPKCDR2back		5′-GCA TCC AGC AGG GCC ACT GGC-3′

TABLE II

Sequences of selected antibody clones

Relevant amino acid positions of antibody clones isolated from the designed synthetic libraries. Positions that are mutated in the primary antibody library are underlined. Residues in H10 and L19, mutated during the affinity maturation procedure, are in boldface. Single amino acid codes are used according to standard IUPAC nomenclature. The sequences of the reported clones have been deposited in the EBI data base. TN-C, human tenascin-C; HCS, human chorionic somatotropin; IFN-G, interferon γ, TeTox, tetanus toxoid.

Antigen	Clone	VH chain			VL chain		
		31–33[a]	50–54[a]	95–98[a]	32[a]	50[a]	91–96[a]
ED-B	A2	SYA	AISGSG	GLSI	Y	G	NGWYPW
	G4	SYA	AISGSG	SFSF	Y	G	GGWLPY
	E1	SYA	AISGSG	PFPY	Y	G	TGRIPP
	H10	**SFS**	**SIRGSS**	PFPY	Y	G	TGRIPP
	L19	**SFS**	**SIRGSS**	PFPY	**F**	**Y**	TGRIPP
TN-C	H7	SYA	AISGSG	PVVP	Y	G	TGRRPM
HCS	17	SYA	AISGSG	FPSF	Y	G	YGVRPH
Factor VIII	H1	SYA	AISGSG	TARA	Y	G	AGLGPR
IFN-G	A4	SYA	AISGSG	RAPA	Y	G	MGDSPT
TeTox	C3	SYA	AISGSG	SLPL	Y	G	WGEKPL

[a] Numbering is according to Ref. 34.

in the order of 1 μg of protein). Immobilization of the antigen on specialty resins or plastic for selections and ELISA assays (4, 6, 8–10) require substantially larger amounts of material.

Panning using biotinylated antigens (10, 24) appears to have several advantages. Performing selections in solution should favor the partial refolding of the antigen of interest. Our experience has shown that good quality libraries can be challenged with antigen concentrations in the 10 nM range and that streptavidin-coated beads can be used for efficient capture of antigen-bound phage particles. Three rounds of panning using a 1-ml volume and a 33-kDa antigen would therefore require: $3 \times 10^{-8} \times 3.3 \times 10^{4} \times 10^{-3} = 10^{-6}$ grams ((rounds of panning) × (concentration) × (molecular weight) × (volume) = grams). In practice, larger amounts of starting material may be required, because of limitations in the efficiency of static elution of the antigen from the gel, biotinylation yield, and protein loss during electrophoretic separation. Biotinylated antigens can also be efficiently immobilized on streptavidin-coated microtiter plates for ELISA screening of individual colonies after selection.

To test the feasibility of the isolation of antibodies against two-dimensional PAGE spots, we chose as a model system a complete fibronectin type III repeat of 91 amino acid residues (the ED-B domain of fibronectin; Ref. 17): a biologically relevant antigen for which antibody performance could be evaluated with technically challenging applications. This protein domain is a marker of angiogenesis (16–18). Recombinant antibodies against the ED-B domain have been used to efficiently detect angiogenesis in cryostat sections of tumors and to target tumoral neovasculature *in vivo* upon intravenous injection (30).

Biotinylated ED-B was eluted from a two-dimensional PAGE spot and used at 10 nM concentration in three rounds of panning, with capture mediated by streptavidin-coated magnetic beads. 0.3 μg of antigen was used in total for the selection. The phage population from the third round of panning was used to infect nonsuppressor HB2151 cells as described (9), and bacterial supernatants obtained from single colonies were used to detect binding to ED-B by ELISA. 32% of tested clones were positive in this assay. The three clones giving the strongest ELISA signal (E1, A2, and G4; Table II) were sequenced and chosen for further characterization.

Antibody Characterization and Performance—ELISA assays were performed using biotinylated ED-B recovered from a gel spot, biotinylated ED-B that had not been denatured, ED-B linked to adjacent fibronectin domains (recombinant 7B89; Ref. 16), and a number of irrelevant antigens. Antibodies E1, A2, and G4 reacted strongly and specifically with all three ED-B containing proteins. This, together with the fact that the three recombinant antibodies could be purified from bacterial supernatants using an ED-B affinity column, strongly suggests that they recognize an epitope present in the native conformation of ED-B. No reaction was detected with fibronectin fragments that did not contain the ED-B domain (data not shown).

To test whether the antibodies isolated against a gel spot had a good affinity toward the native antigen, real-time interaction

TABLE III
Affinities of anti-ED-B scFv fragments

For the high-affinity binders H10 and L19, k_{off} values from BIAcore experiments are not sufficiently reliable due to effects of the negatively charged carboxylated solid dextran matrix; K_d values are therefore calculated from k_{off} measurements obtained by competition experiments (see "Experimental Procedures"). k_{off}, kinetic dissociation constant; k_{on}, kinetic association constant; K_d, dissociation constant. Values are accurate to ±50%, on the basis of the precision of concentration determinations.

Clone	k_{on}	k_{off}[a]	k_{off}[b]	K_d[c]
	$s^{-1}\,M^{-1}$	s^{-1}	s^{-1}	M
A2	1.5×10^5	2.8×10^{-3}		1.9×10^{-8}
G4	4.0×10^4	3.5×10^{-3}		8.7×10^{-8}
E1	1.6×10^5	6.5×10^{-3}		4.1×10^{-8}
H10	6.7×10^4	5.6×10^{-4}	9.9×10^{-5}	1.5×10^{-9}
L19	1.1×10^5	9.6×10^{-5}	6.0×10^{-6}	5.4×10^{-11}

[a] Measured on the BIAcore.
[b] Measured by competition with electrochemiluminescent detection.
[c] $K_d = k_{off}/k_{on}$.

analysis was performed using surface plasmon resonance on a BIAcore instrument (29) as described (30). Monomeric fractions of E1, A2, and G4 scFv fragments bound to ED-B with affinity in the 10^7–10^8 M^{-1} range (Table III).

As a further test of antibody specificity and usefulness, a two-dimensional PAGE immunoblot was performed, running on gel a lysate of the human melanoma cell line COLO-38, to which minute amounts of the ED-B containing recombinant 7B89 protein had been added (Fig. 2). ScFv(E1) stained strongly and specifically only the 7B89 spot.

Antibodies E1, A2, and G4 were used to immunolocalize ED-B containing fibronectin (B-FN) in cryostat sections of glioblastoma multiform, an aggressive human brain tumor with prominent angiogenic processes. Fig. 3 shows serial sections of glioblastoma multiform, with the typical glomerulus-like vascular structures stained in red by the three antibodies.

Affinity Maturation of an Anti-ED-B Antibody—ScFv(E1) was selected to test the possibility of improving its affinity with a limited number of mutations of CDR residues located at the periphery of the antigen binding site (Fig. 1*A*). We combinatorially mutated residues 31–33, 50, 52, and 54 of the antibody VH and displayed the corresponding repertoire on filamentous phage. These residues are found to frequently contact the antigen in the known three-dimensional structures of antibody-antigen complexes. The resulting repertoire of 4×10^8 clones was selected for binding to the ED-B domain of fibronectin. After two rounds of panning, and screening of 96 individual clones, an antibody with 27-fold improved affinity was isolated (H10; Tables II and III). Similarly to what others have observed with affinity-matured antibodies, the improved affinity was because of slower dissociation from the antigen, rather than by improved k_{on} values (41–43). The antibody light chain is often thought to contribute less to the antigen binding affinity as supported by the fact that both natural and artificial antibodies devoid of light chain can still bind to the antigen (44, 45). For this reason we chose to randomize only two residues (32 and 50) of the VL domain, which are centrally located in the antigen binding site (Fig. 1*A*) and often found in three-dimensional structures to contact the antigen. The resulting library, containing 400 clones, was displayed on phage and selected for antigen binding. From analysis of the dissociation profiles using real-time interaction analysis with a BIAcore instrument (29) and k_{off} measurements by competition experiments with electrochemiluminescent detection (Fig. 4; see "Experimental Procedures"), a clone (L19) was identified that bound to the ED-B domain of fibronectin with a K_d = 54 pM (Tables II and III).

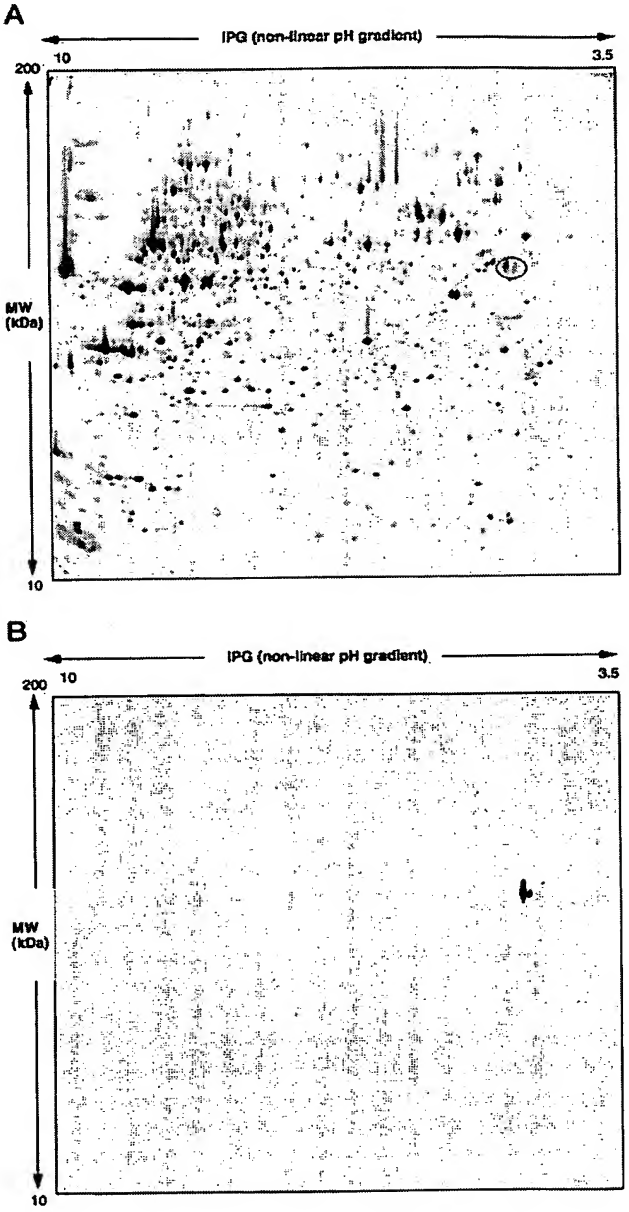

FIG. 2. **Two-dimensional gels and Western blotting.** *A*, silver staining of the two-dimensional PAGE of a lysate of human melanoma COLO-38 cells, to which recombinant ED-B-containing 7B89 had been added. The two 7B89 spots (*circle*) are because of partial proteolysis of the His tag used for protein purification. *B*, immunoblot of a gel, identical to the one of *A*, using the anti-ED-B E1 (Table II) and the M2 anti-FLAG antibodies as detecting reagent. Only the 7B89 spots are detected, confirming the specificity of the recombinant antibody isolated from a gel spot. *IPG*, immobilized pH gradient.

DISCUSSION

By incorporating principles of protein design, we have constructed a large repertoire of functional antibodies, which can further be improved using a general and rapid affinity maturation strategy.

The use of antibody phage libraries has shown that the most frequently used antibody germ line segments in human repertoires are often selected also from synthetic repertoires (10).

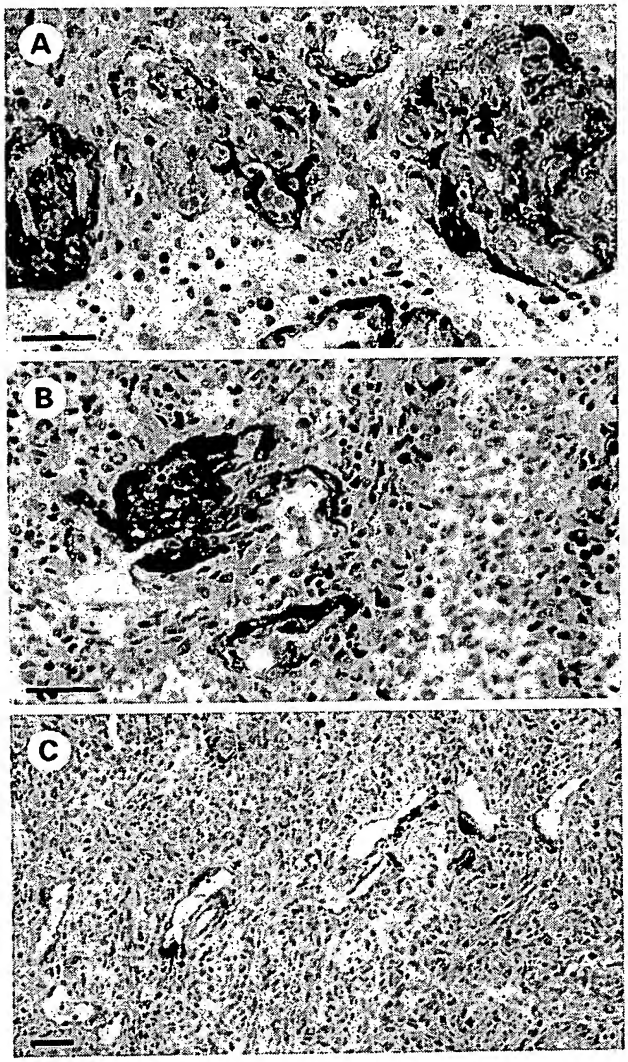

FIG. 3. **Immunohistochemistry.** Immunohistochemical experiments on serial sections of glioblastoma multiform showing the typical glomerulus-like vascular structures stained using scFvs E1 (*A*), A2 (*B*), and G4 (*C*). *Scale bars*: 20 μm.

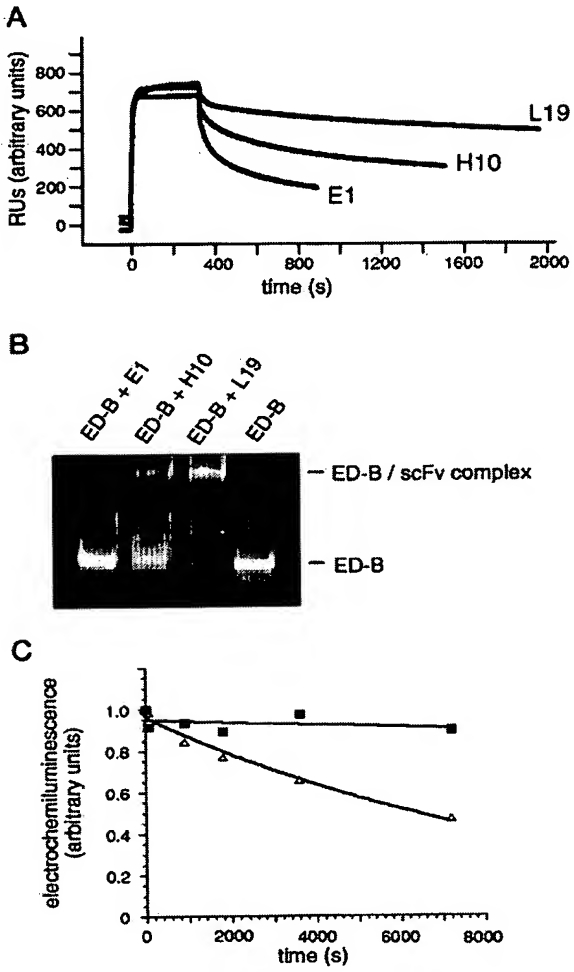

FIG. 4. **Stability of antibody·ED-B complexes.** Analysis of the binding of scFvs E1, H10, and L19 to the ED-B domain of fibronectin. *A*, BIAcore sensograms showing the improved dissociation profiles obtained upon antibody affinity maturation. *B*, native gel electrophoretic analysis of scFv·ED-B complexes. Only the high-affinity antibody L19 can form a stable complex with the fluorescently labeled antigen. Fluorescence detection was performed as described (47). *C*, competition of the scFv·ED-B-biotin complex, with a 100-fold molar excess of unbiotinylated ED-B, monitored by electrochemiluminescence using an Origen apparatus. A long half-life for the L19·ED-B complex can be observed. *Black squares*, L19; *open triangles*, H10.

Furthermore, synthetic antibody repertoires constructed with a single germ line segment have reliably yielded good binders against a large variety of antigens (9, 16, 33, 46).[2] For these reasons, we have used only the germ line segments DP47 and DPK22 (19, 20, 34–36) for the synthetic antibody library construction.

The choice to mutate only CDR3 loops in our repertoire was dictated by the following considerations. CDR3s of VH and VL are centrally located in the antigen binding site (Fig. 1), a geometric property paralleled by their role in determining the antigen-binding affinity and by the high diversity of CDR3 sequences observed in natural antibody repertoires. In all the three-dimensional structures of antibody-antigen complexes known so far, at least one residue of the CDR3 loops, but not necessarily of CDR1 and CDR2, is in contact with the antigen. In nature the length of CDR3 can vary from few residues to more than 20 residues (10). However, because very high affinity antibodies can be obtained with short CDR3 in the heavy chain (48), we opted for short CDR3s to limit the potential diversity of the library and reduce clone to clone variability. The short CDRs used in our library design may facilitate the modeling of antigen recognition by the antibody using a computational approach (49). Furthermore, the use of short CDR3s in VH and VL immunoglobulin domains is generally associated with better antibody stability to proteolysis and improved binding and bacterial expression (18, 21, 22).[3]

The selection of antibodies from primary repertoires, followed by mutagenesis of CDRs and phage display selection, allows the isolation of binders with improved affinity (30, 33, 41, 42). The anti-ED-B scFv(L19), obtained by combinatorial

[2] P. M. Kirkham, D. Neri, and G. Winter, submitted for publication.

[3] A. Pini, F. Viti, A. Santucci, B. Carnemolla, L. Zardi, P. Neri, and D. Neri, unpublished observations.

mutagenesis of judiciously selected CDR residues is one of the highest affinity antibodies isolated from phage display libraries. Thanks to the modular design of the antibody library consisting of a single germ line segment for both VH and VL, the maturation strategy did not rely on sequencing. The same degenerate oligonucleotide primers used for L19 should be applicable for the maturation of other antibodies isolated from the same library. Only few positions had to be randomized to achieve significant improvements in binding affinity. Previous work had shown that antibodies derived from natural repertoires required substantially more sequencing, mutagenesis, and cycles of panning of phage display libraries (41, 42) to achieve comparable levels of binding affinity. Unlike what has been described by other authors, we did not find that several cycles of stringent selections were necessary for the isolation of very high-affinity binders from antibody repertoires on phage (41, 42, 50). Our affinity maturation libraries, selected in parallel with different biopanning strategies, rapidly yielded improved binders when selected on antigen-coated solid supports, but not when selected with the stringent kinetic methodology of Low *et al.* (Ref. 50; data not shown).

Markers of angiogenesis are becoming increasingly popular for immunotherapy of cancer and other diseases associated with vascular proliferation (51). They are expressed in the majority of aggressive solid tumors and should be readily accessible to specific binders injected intravenously (51, 52). Occlusion of the neovasculature may result in tumor infarction and collapse (53, 54), and antibody affinity appears to play a role in determining tumor targeting efficiency (30). Experiments are in progress, which are aimed at evaluating the tumor targeting properties of scFv(L19), an antibody isolated against a protein eluted from a gel spot.

The introduction of two-dimensional electrophoresis with immobilized pH gradients (for review, see Refs. 55 and 56) has produced significant improvements in the resolution, reproducibility, and amounts of proteins that can be used in two-dimensional PAGE. The use of minute amounts (<1 μg) of biotinylated antigens eluted from two-dimensional gels is sufficient for the isolation of recombinant antibodies from phage display libraries. These antibodies can recognize the antigen in the native conformation and have proved to be useful reagents in a number of immunochemical techniques, including ELISA, two-dimensional immunoblotting, and immunohistochemistry. Because selections with biotinylated antigens are performed in solution, partial protein renaturation may occur after elution from the gel. This was observed in the case of the ED-B domain of fibronectin. Whereas it should be possible to biotinylate the protein sample after electrophoretic separation, prebiotinylation is likely to be a more convenient approach. Suitable treatment of the biological sample may lead to the biotinylation of only a subset of total protein content (*e.g.* extracellular domains of membrane proteins). Reaction of standard biotinylating reagents with lysine residues, however, converts a positively charged side chain into an uncharged amide. It should be possible, however, to design biotinylating reagents that incorporate a positive charge (*e.g.* a tertiary amine) with a pK_a similar to the one of lysine side chains. Such reagents should increase only minimally the molecular weight of the labeled protein, while not perturbing its isoelectric point.[4]

Additional advantages of raising phage antibodies against gel spots include the possibility of obtaining several affinity reagents against unpurified antigens contained in the protein mixture applied to the gel.

[4] L. Lozzi, A. Pini, P. Neri, and D. Neri, manuscript in preparation.

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